Proteomic and metabolomic responses to connexin43 silencing in primary hepatocyte cultures

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IN VITRO SYSTEMS

Proteomic and metabolomic responses to connexin43 silencing in primary hepatocyte cultures

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Abstract Freshly established cultures of primary hepatocytes progressively adopt a foetal-like phenotype and display increased production of connexin43. The latter is a multifaceted cellular entity with variable subcellular locations, including the mitochondrial compartment. Cx43 forms hemichannels and gap junctions that are involved in a plethora of physiological and pathological processes, such as apoptosis. The present study was conducted with the goal of shedding more light onto the role of connexin43 in primary hepatocyte cultures. Connexin43 expression was suppressed by means of RNA interference technology, and the overall outcome of this treatment on the hepatocellular proteome and metabolome was investigated using tandem mass tag-based differential protein profiling and ^{1}H NMR spectroscopy, respectively. Global protein profiling revealed a number of targets of the connexin43 knock-down

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procedure, including mitochondrial proteins (heat shock protein 60, glucose-regulated protein 75, thiosulphate sulphurtransferase and adenosine triphosphate synthase) and detoxifying enzymes (glutathione S-transferase μ 2 and cytochrome P450 2C70). At the metabolomic level, connexin43 silencing caused no overt changes, though there was some evidence for a subtle increase in intracellular glycine quantities. Collectively, these data could further substantiate the established existence of a mitochondrial connexin pool and could be reconciled with the previously reported involvement of connexin43 signalling in spontaneously occurring apoptosis in primary hepatocyte cultures.

Keywords Primary hepatocyte · Connexin43 · Proteomics · Metabolomics

Abbreviations

ATP	Adenosine triphosphate
Cx	Connexin
DDA	Data-dependent acquisition
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GO	Gene ontology
GRP75	Glucose-regulated protein 75
HSP60	Heat shock protein 60
IPI	International protein index
LC/MS	Liquid chromatography/mass spectrometry
LOWESS	Locally weighted scatterplot smoothing
LTQ	Linear ion trap
NMR	Nuclear magnetic resonance
OT	Orbitrap
PbAE2	1,6-Hexanediol diacrylate-based poly-beta-aminoester
PBS	Phosphate-buffered saline solution
RF	Relative frequence
SDS–PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
TBS	Tris-buffered saline solution
TMT	Tandem mass tags
TSP	Trimethylsilyltetradeuteropropionic acid

Introduction

Connexin (Cx) proteins and their channels constitute three levels of cellular communication. First, connexins assemble into hexameric structures, called hemichannels, which dock in pairs at the cell plasma membrane surface to generate gap junctions between neighbouring cells. Gap junctional intercellular communication implies the passive diffusion of small and hydrophilic substances, including glucose, glutamate, glutathione, adenosine triphosphate (ATP), cyclic adenosine monophosphate, inositol trisphosphate and ions. Undocked hemichannels create a second platform for communication, by connecting the cytosol with the extracellular environment and by conveying essential metabolites, like ATP, nicotinamide adenine dinucleotide, glutamate, prostaglandins and glutathione. Hemichannels can also reside at atypical subcellular locations, such as in mitochondria, where they are thought to serve as a conduit for ion fluxes. A third level of communication is formed by connexin proteins themselves, which can affect gene expression patterns independently of gap junction or hemichannel functionality (Decrock et al. 2009; Vinken et al. 2006a, 2011a). The interplay between these three cellular signalling routes is not only of major importance for the maintenance of tissue homeostasis, but also fulfils a key function in a variety of pathological events, including cell death, inflammation, oxidative stress and ischaemic insults (Decrock et al. 2009).

Cultures of primary hepatocytes are prominent in vitro models in pharmacotoxicology (Elaut et al. 2006; Hewitt et al. 2007). However, they are prone to dedifferentiation, which is caused, at least in part, through the induction of an inflammatory response during their isolation from the freshly acquired liver tissue and which is followed by the onset of cell death during their subsequent cultivation (Elaut et al. 2006; Vinken et al. 2006b, 2011b). This deteriorative process is associated with a transition in connexin expression. Indeed, in the adult liver, hepatocytes abundantly produce Cx32, but in culture, they switch to Cx43 (Vinken et al. 2006b). The biological relevance of induced hepatocellular Cx43 expression in primary hepatocyte cultures, a process also observed in vivo during hepatocarcinogenesis (Vinken et al. 2008), remains obscure. We previously showed that Cx43 is not involved in the loss of the differentiated phenotype per se (Vinken et al. 2012a), but rather plays a permissive role in spontaneous apoptosis in primary hepatocyte cultures by involving both gap junction and hemichannel activity (Vinken et al. 2012b). In the current study, we investigate whether Cx43 protein as such can affect hepatocellular protein production and endogenous metabolism in vitro at a global scale. To this end, Cx43 expression was suppressed in primary hepatocyte cultures through RNA interference technology followed by evaluation of the resulting proteomic and metabolomic profiles.

Materials and methods

Chemicals and reagents

The 1,6-hexanediol diacrylate-based poly-beta-aminoester (PbAE2) was synthesized as described elsewhere

(Vandenbroucke et al. 2008). For liquid chromatography (LC)/mass spectrometry (MS) analysis, all solvents and additives were of ultragrade performance LC grade quality (Biosolve, the Netherlands). All other chemicals were commercially available products of analytical grade and were supplied by Sigma (Belgium), unless specified otherwise.

Hepatocyte isolation and cultivation

Procedures for the housing of rats, and isolation and cultivation of hepatocytes were approved by the local ethical committee of the Vrije Universiteit Brussel (Belgium). Male outbred Sprague–Dawley rats (Charles River Laboratories, Belgium) were kept under controlled environmental conditions with free access to food and water. Hepatocytes were isolated using a two-step collagenase method, including purification by serial differential centrifugation, and cell viability was assessed by trypan blue exclusion (Papeleu et al. 2006). Viable (≥85 %) hepatocytes were plated at a density of 0.56×10^5 cells/cm^2 in William's medium E (Invitrogen, Belgium) supplemented with 7 ng/ml glucagon, 292 mg/ml L-glutamine, antibiotics (7.33 I.E./ml sodium benzyl penicillin, 50 μg/ml kanamycin monosulphate, 10 μg/ml sodium ampicillin and 50 μg/ml streptomycin sulphate) and 10 % foetal bovine serum. After 4 h, 1 day, 2 days and 3 days, the cell culture medium was removed and replaced by serum-free medium supplemented with 25 μg/ml hydrocortisone sodium hemisuccinate and 0.5 μg/ml insulin ("hepatocyte culture medium"). Samples were taken on day 1 and day 4 of the cultivation period.

Small interfering RNA transfection

Cx43 expression was suppressed by small interfering RNA (siRNA) treatment with On-target plus smart pool siRNA from Dharmacon (Belgium), which contains four different siRNA duplexes directed against rat *gja1* (sense strand sequences: siRNA number 1: 5′-CAACAACCUGGCUGC GAAAUU-3′; siRNA number 2: 5′-UGAUUGAAAUGUC GAGUUAUU-3′; siRNA number 3: 5′-CGUGAAGGGAA GAAGCGAUUU-3′; siRNA number 4: 5′-UUACUGAGA UUCUGCGAUAUU-3′). Preparation of PbAE2/siRNA complexes was carried out as previously described (Vandenbroucke et al. 2008; Vinken et al. 2012a, b). The siRNA transfection was started 20 h after cell seeding by replacing the initial cell culture medium by hepatocyte culture medium, containing the PbAE2/siRNA complexes (final siRNA concentration 100 nM). After 5 h, the cell culture medium was removed and replaced by regular hepatocyte culture medium. The hepatocyte culture medium was renewed another two times (19 h and 43 h post-transfection), and sampling was performed 72 h post-transfection (i.e., at day 4 of the cultivation period). Experiments with non-targeting siRNA (Dharmacon, Belgium) were performed in parallel.

Proteomics analysis

For the preparation of total protein lysates, hepatocytes were harvested from cell culture plates by scraping and washed twice with cold phosphate-buffered saline solution (PBS). Pelleted cells were lysed by addition of radio-immunoprecipitation lysis buffer (Thermo Fisher Scientific, Germany) complemented with 1 % phosphatase inhibitor (Thermo Fisher Scientific, Germany) and 1 % protease inhibitor (Thermo Fisher Scientific, Germany). Following controlled sonication and cooled (4 °C) centrifugation for 5 min at 14,000×*g*, the supernatant was used for protein concentration determination using a BCA Protein Assay kit (Pierce, Belgium). Samples were spiked with 1 % casein-β and 1 % Rapigest SF Surfactant (Waters, Belgium), incubated at 100 °C for 5 min, mixed and chilled at 4 °C. Equal amounts of protein extracts obtained from three biological replicas were pooled. Each pooled sample was subjected to acetone-based protein precipitation followed by in-solution tryptic proteolysis. The resulting digest was prepared for isotopic peptide labelling using tandem mass tag (TMT) kits and reagents (Thermo Fisher Scientific, Germany) according to the manufacturer's protocol. In addition to the three experimental conditions (i.e., Cx43 siRNA, non-targeting siRNA and control), an additional sample was reconstituted from equal amounts of the three experimental conditions and was used for normalization purposes during quantitative data analysis. The final multiplexed sample was reconstituted from equal amounts of each labelled condition and subjected to three replicate LC/MS analyses. Nanoflow LC/MS/MS was performed on a dual binary channel nanoLC Ultra 2D system (Eksigent, United States of America) connected to a linear ion trap (LTQ) Velos Orbitrap (OT) mass spectrometer (Thermo Fisher Electron, Germany), equipped with a Triversa chip-based electrospray source (Advion Biosystems, United States of America) operating at 1.8 kV. A volume of 10 μL, representing a predigest protein load of 1 μg, was loaded on a C18 precolumn (PepMap 100, 5 μm particles, 20 mm × 200 μm internal diameter, Dionex, United States of America). After 5 min, the trapping column was switched in line with an analytical C18 column (Acclaim Pepmap 100, 3 μm particles, 150 mm × 75 μm internal diameter) (Dionex, United States of America). Gradient elution of peptides was accomplished during a 70-min run. Peptide MS spectra were recorded in the OT with a resolution of 60,000 at m/z 400. The maximum injection time was set to 500 ms for both LTQ and OT, and lock mass was enabled with polysiloxane ion at m/z 445.12024. Up to five co-eluting peptide masses were selected in data-dependent acquisition (DDA) mode for interrogation on tandem MS. Recurrent masses were

dynamically excluded from DDA selection during 30 s. The set of selected peptides was analysed by two different mass spectral settings to ascertain an optimal information retrieval. First, a low-energy collision- induced dissociation regime in the LTQ analyser was applied to generate reliable and complementary peptide sequence fragmentation data. Secondly, high-collision energy fragmentation regime in the OT analyser was applied in order to establish reliable TMT reporter ions for robust quantification (Kocher et al. 2009). Raw files of the three replicate analyses were processed via the Proteome Discoverer work flow manager version 2.0 (Thermo Fisher Scientific, United States of America). For peptide identification, fragmentation spectra from both analysers were used for database searches against international protein index (IPI) rat (ipi.RAT.v3.87 containing 39,925 dbase entries) (Kersey et al. 2004) using Sequest version 1.0.43 embedded in Proteome Discoverer 2.0. Carbamidomethylation and the modification of the *N*-termini and lysine by the TMT-reactive group were set as fixed modifications, while oxidation was set as variable modification. Peptide mass tolerance was set at 5 ppm, and fragment mass tolerance was set to 0.5 Da and 20 millimass units for the LTQ and OT, respectively. Two missed cleavages were allowed. For confident and high-confident peptide identification, a threshold scheme based on the Sequest Xcorr score was used. The thresholds depend on the charge state of the fragmented molecular ion (Supplementary Table S1). This resulted in a final total of 1,790 rat proteins identified. A gene ontology (GO) analysis of the proteins showed that all major cellular components were sampled (data not shown) and indicated that the sampled set of proteins did not suffer from any major sample preparation bias. A comparative GO profile of the identified hepatocyte proteins was compared with the total rat GO profile and indicated significant enrichment or depletion for hepatocyte-specific cellular components, cellular functions and metabolic processes (Supplementary Table S2). The false discovery rate was assessed by a target-decoy approach using the reversed sequences of IPI rat. By using the threshold scheme, false discovery rate values of 0.0487 and 0.0081 were obtained for the confident and high-confident peptides, respectively. This reporter ion information was extracted from the tandem MS with a mass tolerance of 20 ppm. In the case multiple ions were present within the mass tolerance, the most confident peak was selected. Reporter intensities were normalized to remove systematic effects and possible confounders using a locally weighted scatterplot smoothing (LOWESS) normalization (Yang et al. 2002).

Immunoblotting

For the preparation of total protein lysates, hepatocytes were harvested from cell culture plates by scraping and washed twice with cold PBS. Cells were homogenized in lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM ethylenediamine tetra-acetic acid, 2.5 mM ethyleneglycol tetra-acetic acid, 0.1 % Tween 20 and 10 % glycerol) supplemented with 0.1 mM phenylmethylsulphonyl-fluoride, 5 mM dithiothreitol and protease inhibitor cocktail (Roche, Germany). Following sonication for 10 s, samples were left on ice for 1 h. Cell lysates were centrifuged at $13{,}791 \times g$ for 5 min, and protein concentrations were determined in the supernatants according to the Bradford procedure (Bradford 1976) using a Bio-Rad protein assay kit (Bio-Rad, Germany). Proteins were fractionated on sodium dodecyl sulphate polyacrylamide (SDS–PAGE) and blotted afterwards onto nitrocellulose membranes (Amersham, UK). Blocking of the membranes was performed with 5 % non-fatty milk in Tris-buffered saline solution (TBS; 20 mM Tris, 135 mM NaCl) containing 0.1 % Tween 20. Membranes were incubated overnight at 4 °C with a primary antibody directed against Cx32, Cx43, heat shock protein 60 (HSP60) or glucose-regulated protein 75 (GRP75) (Table 1), followed by incubation for 1 h at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies (Dako, Denmark). Excessive antibody was removed by washing the membranes in Tween-supplemented TBS. The proteins were detected by means of an enhanced chemiluminescence Western blotting system (Amersham, United Kingdom). For semi-quantification of the results, blots were further incubated with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Abcam, United Kingdom). Blots were scanned, and densitometric analyses were performed by using the Quantity One software (Bio-Rad, Germany). For Cx43, marker signals in the non-targeting siRNA and the Cx43 siRNA conditions were normalized to the corresponding GAPDH signals and were expressed as percentage of the normalized marker signals in the untreated control condition. For HSP60 and GRP75, marker signals in the Cx43 siRNA condition were normalized to the corresponding GAPDH signals and were expressed as percentage of the normalized marker signals in the non-targeting siRNA condition.

Table 1 Primary antibodies used for immunoblot analysis

Antigen	Supplier	Reference	Species	Type	Dilution
Cx32	Sigma	C3470	Rabbit	Polyclonal	1/500
Cx43	Sigma	C6219	Rabbit	Polyclonal	1/4,000
HSP60	Santa Cruz	sc13966	Rabbit	Polyclonal	1/1,000
GRP75	Santa Cruz	sc13967	Rabbit	Polyclonal	1/1,000
GAPDH	Abcam	ab8245	Mouse	Monoclonal	1/20,000

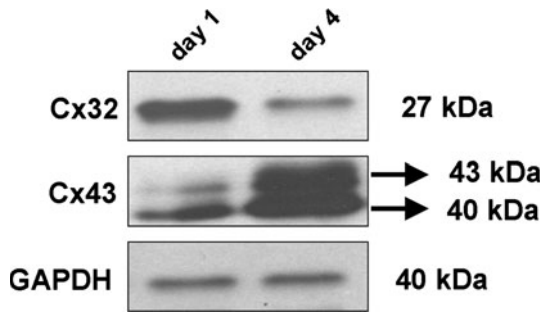

Fig. 1 Connexin expression in primary hepatocyte cultures. Freshly isolated rat hepatocytes were cultivated in a monolayer configuration. Samples were taken on day 1 and day 4 of the cultivation period and subjected to immunoblot analysis as described in the "Materials and methods" section, using primary antibodies that specifically recognize Cx32 and Cx43 (Table 1). *Blot* shown is a representative of three independent experiments

Metabolomics analysis

Intracellular (i.e., aqueous cell fractions) and extracellular (i.e., cell culture media) samples of the primary rat hepatocyte cultures were prepared as previously described (Ellis et al. 2011). Aqueous soluble metabolites (i.e., intracellular fractions) were isolated from hepatocytes using a chloroform/methanol/water extraction method. After fractionation, the samples were allowed to evaporate at room temperature for about 12 h to remove any organic solvent, and the remainder was freeze-dried. The lyophilized sample was then reconstituted in 600 µL of phosphate buffer and centrifuged for 5 min at 16,000×*g* before being transferred to a standard 5-mm glass nuclear magnetic resonance (NMR) tube. The cell culture media (i.e., extracellular fractions) were prepared for spectroscopic analysis by adding 50 µL of D_2O containing 0.2 % trimethylsilyltetradeuteropropionic acid (TSP) to 550 µL of cell culture media sample, of which 550 µL was transferred to a standard 5-mm NMR tube. All reagents were checked prior to sample preparation by obtaining 1D ^{1}H NMR spectra to ensure that they contained no contaminants that could interfere with the downstream spectroscopic analysis. For NMR spectroscopy, high-resolution 1D ^{1}H NMR spectra of intracellular and extracellular samples were acquired at 14.1 T (600.13 MHz ^{1}H frequency) using a Bruker Avance 600 spectrometer (Bruker Biospin, Germany) fitted with a 5-mm tube probehead and a Bacs 60 automated sample changer (Bruker BioSpi, Germany). Acquisition of spectra was controlled using Xwin-NMR and Icon-NMR (Bruker Biospin, Germany). Automatic shimming was used to improve the magnetic field homogeneity prior to the acquisition of spectral data for each sample. Carr-Purcell-Meiboom-Gill ^{1}H spectra were obtained using the pulse sequence (RD-90°-[τ-180°-τ]n-AQ). The

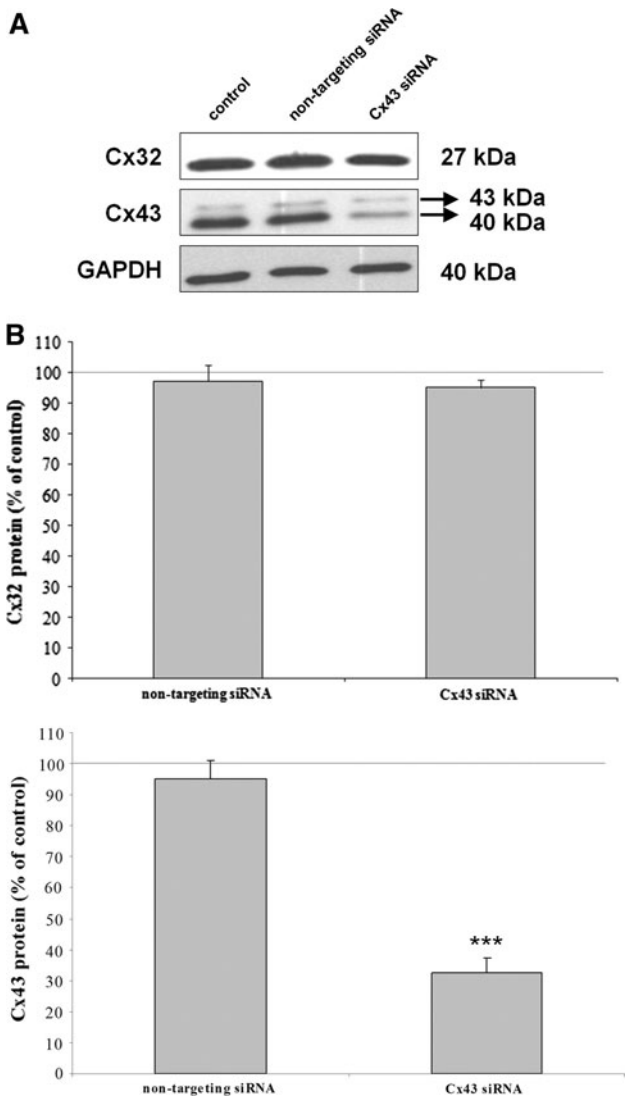

Fig. 2 Cx43 suppression in primary hepatocyte cultures. **a** Freshly isolated rat hepatocytes were cultivated in a monolayer configuration. Twenty hours after plating, cell cultures were transfected with 100 nM non-targeting siRNA or Cx43 siRNA for 5 h as described in the "Materials and methods" section. Samples were taken 72 h post-transfection and subjected to immunoblot analysis as described in the "Materials and methods" section, using a Cx32 or a Cx43 primary antibody (Table 1). **b** For semi-quantification of the results, Cx32 or Cx43 signals in the non-targeting siRNA and the Cx43 siRNA conditions were normalized to the GAPDH corresponding signals and were expressed as percentage of the normalized Cx32 (*upper panel*) or Cx43 (*lower panel*) signals, respectively, in the control condition (indicated with a *dashed line*). Data were expressed as mean ± SD of four independent experiments. Results were evaluated by one-way analysis of variance followed by post hoc Bonferroni tests. *Asterisks* indicate significant differences compared with the non-targeting siRNA condition (***$p < 0.001$)

fixed echo time (τ) was set at 400 µs, with a total spin-echo time of 64 ms. During the acquisition period (AQ, 2.73 s), the free induction decay was recorded into 64 k datapoints in the time domain, with a spectral width of 20 ppm.

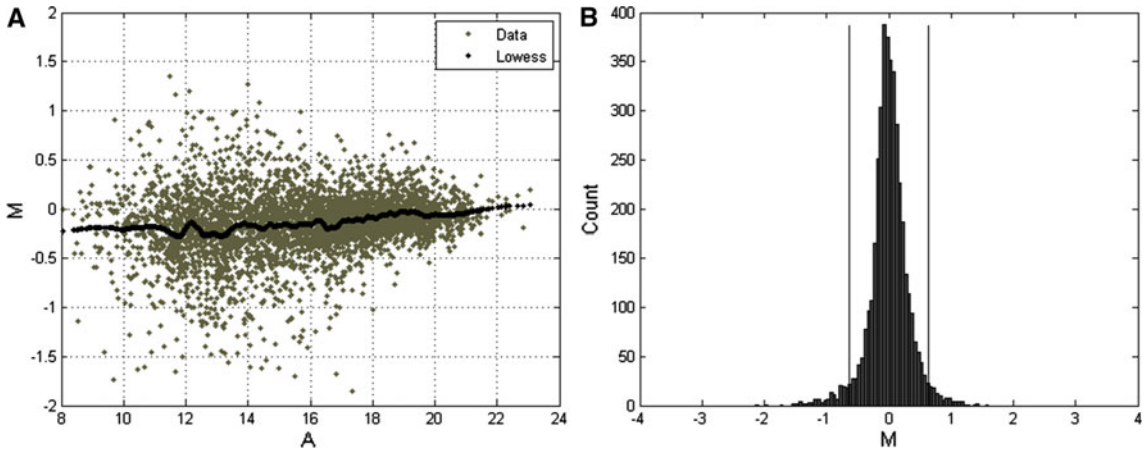

Fig. 3 Effect of Cx43 suppression on the proteome in primary hepatocyte cultures. Freshly isolated rat hepatocytes were cultivated in a monolayer configuration. Twenty hours after plating, cell cultures were transfected with 100 nM non-targeting siRNA or Cx43 siRNA for 5 h as described in the "Materials and methods" section. Samples were taken 72 h post-transfection and subjected to proteomics analysis as described in the "Materials and methods" section. **a** Data are shown in a MA-plot indicating the log2 ratio (M) versus average log2 intensity (A) of the non-normalized reporter intensities of the Cx43 siRNA and non-targeting siRNA conditions. The *dots* indicate the results of the LOWESS regression. **b** Data are shown in a histogram of the log2 ratio after LOWESS normalization. The *vertical lines* indicate the 95 % CI

Typically, spectra were recorded as the sum of 128 transients following 16 dummy scans. In both cases, suppression of the water resonance centred at δH 4.7 ppm was achieved by the application of a presaturation pulse during the relaxation delay. A line broadening of 0.3 Hz was applied to each spectrum. Assignment of resonances to specific metabolites was based on the Chenomx profiler in the NMR suite 6.1 software (Chenomx Inc., Canada) and in-house assignment databases. The assignment of intracellular glycine was confirmed by addition of a pure standard at a known concentration to the biological samples.

Statistical analysis

For immunoblot analyses, data were expressed as mean ± SD of at least three independent experiments. Results were evaluated by one-way analysis of variance followed by post hoc Bonferroni tests. For the metabolomic analyses, NMR spectral data were imported and processed in Matlab (Mathworks) using in-house software compiled by Dr. T.M.D. Ebbels, Dr. H.C. Keun, Dr. J.T. Pearce and Dr. R. Cavill. ^{1}H NMR spectra were automatically phased, baseline corrected, and referenced and normalized to the TSP resonance at δ 0. The spectra were then normalized using a median fold change normalization (Dieterle et al. 2006) before subsequent analysis. In total, 34 metabolites were assigned, and the peak edges of the least overlapped peaks were manually defined for integration. Differences between conditions were assessed using a homoscedastic t-test.

Results

Connexin expression in primary hepatocyte cultures

Hepatocytes mainly harbour Cx32 in the adult liver (Vinken et al. 2008). However, during isolation and cultivation of primary hepatocytes, Cx32 steady-state protein levels drastically decline, with only marginal Cx32 immunoreactivity at the end of the 4-day cultivation period (Fig. 1). A number of mechanisms could underlie this deterioration of Cx32 production, including oxidative stress, which is triggered during the hepatocyte isolation procedure (Elaut et al. 2006). Simultaneously, Cx43, a connexin species only expressed by foetal hepatocytes and by adult non-parenchymal cells in vivo (Vinken et al. 2008), becomes gradually detectable. Cx43 hereby is observed as a dual-band signal, representing non-phosphorylated Cx43 (40 kDa; lower band) and phosphorylated Cx43 (43 kDa; upper band), of which the former is predominantly present in primary hepatocyte cultures (Fig. 1). The molecular mechanisms that drive this process are unclear, though a recent study shows that Wnt signalling and hence altered transcriptional regulation could explain Cx43 appearance in primary hepatocyte cultures (Yamaji et al. 2011).

Cx43 suppression in primary hepatocyte cultures

RNA interference technology was used to investigate the role of Cx43 in primary hepatocyte cultures. For this purpose, four siRNA duplexes directed against Cx43 mRNA

Table 2 Candidate proteins for the validation of the proteomics analysis

Log2 ratio	Dbase entry	Description
−1.6	IPI00197129.1	Actin, aortic smooth muscle
	IPI00189819.1	Actin, cytoplasmic 1
	IPI00194087.3	Actin, α-cardiac muscle 1
	IPI00896224.1	Actin, cytoplasmic 2
	IPI00200455.1	Actin, γ-enteric smooth muscle
	IPI00189813.1	Actin, α-skeletal muscle
	IPI00560160.2	43-kDa protein
	IPI00948496.1	33-kDa protein
	IPI00958161.1	Actin, γ-1 propeptide-like
	IPI00960010.1	β-actin FE-3 (fragment)
−1.5	IPI00339148.2	60-kDa heat shock protein, mitochondrial
−1.4	IPI00411230.3	Glutathione S-transferase μ 2
−1.4	IPI00366293.3	Thiosulphate sulphurtransferase
	IPI00952474.1	Uncharacterized protein
−1.2	IPI00389571.6	Keratin, type II cytoskeletal 8
−1.0	IPI00188804.1	60S acidic ribosomal protein P2
	IPI00388179.1	Uncharacterized protein
	IPI00734740.2	Uncharacterized protein
1.0	IPI00778443.1	Cytochrome P450 2C70
1.0	IPI00551812.1	ATP synthase subunit-β, mitochondrial
1.0	IPI00231555.1	Isoform PYBP1 of polypyrimidine tract-binding protein 1
	IPI00390239.1	Polypyrimidine tract-binding protein 1 protein
	IPI00390801.3	Uncharacterized protein
	IPI00561640.2	Uncharacterized protein
	IPI00562850.2	59-kDa protein
1.1	IPI00206624.1	78-kDa glucose-regulated protein
1.4	IPI00363265.3	Stress-70 protein, mitochondrial

Hepatocyte proteins, identified based on peptide homology, which showed at least a twofold change in relative abundance upon treatment with Cx43 siRNA are considered suitable for further validation. Entries and protein descriptions are obtained from the IPI rat database

were introduced into the hepatocytes by means of PbAE2, a cationic biodegradable poly-beta-aminoester (Vandenbroucke et al. 2008). Transfections with non-targeting siRNA, bearing at least four mismatches with known rat genes, were performed in parallel in order to check the specificity of the method. Semi-quantitative immunoblot analysis indicated a significant ($p < 0.001$) reduction in Cx43 protein amounts to 32.5 ± 4.7 % of the control level in primary hepatocyte cultures exposed to Cx43 siRNA, while Cx43 levels remained statistically unchanged in non-targeting siRNA-treated cultures. Cx32 levels were unaffected by Cx43 siRNA, thus showing the specificity of the method (Fig. 2a, b).

Effect of Cx43 suppression on the proteome in primary hepatocyte cultures

Proteomics analysis was performed on samples taken from primary hepatocyte cultures treated with Cx43 siRNA or non-targeting siRNA as well as from their untreated counterparts. Figure 3a displays the result of the normalization on the log2 of the ratio between the Cx43 siRNA-treated and non-targeting siRNA-treated reporter ions. Scattered points are centred at the zero line, indicating that there is no systematic bias in the quantification channels. Furthermore, the paired abundance measures exhibit a clear drop shape, which suggests that the variability changes with the overall intensity level. From the population of 4,119 tandem MS spectra, 2.5 % upregulated and down-regulated peptides were selected. The vertical lines in Fig. 3b visualize the 95 % interval of reporter ion ratios between the targeting sample and non-targeting sample. Ratios that fell outside the interval were considered potential candidates for optional investigation by Western blot analysis. Only high-confident peptide identifications were considered for further investigation.

In order to examine the effect of Cx43 suppression on the overall proteome profile of the cultured hepatocytes, a limited GO profile analysis was performed (Supplementary Table S3). For that purpose, only the identified proteins that were characterized with at least a 1.5-fold change in relative abundance compared to the control sample were used. It should be noted that peptide ratios were adjusted by median normalization. In addition, the selected proteins have an abundance variability of less than 30 %, and the selected proteins were not present in the list of significant altered proteins of the non-targeting siRNA-treated cells. This resulted in a final set of 27 proteins (Supplementary Table S4). In total, 25 proteins were changed in the non-targeting siRNA-treated cells versus the control condition (Supplementary Table S5). From this exercise, it was clear that the most affected cellular compartment upon Cx43 suppression is the mitochondrion, followed by the cytoplasm and the cellular membrane system. With respect to cellular function and processes, this is reflected in the metal ion–binding protein set, including members of the cytochrome protein family, the actin and actin-binding protein family, chaperones, redox proteins and stress responsive proteins (Supplementary Table S3). A list of 11 candidate proteins for validation of the proteomics analysis was selected thereof (Table 2). A number of these proteins were studied in more detail by means of Western blot analysis (Fig. 4). Since the log2 ratio data showed that mitochondrial 60-kDa heat shock protein (HSP60) and mitochondrial stress-70 protein/glucose-regulated protein 75 (GRP75) were among the most prominent negative and

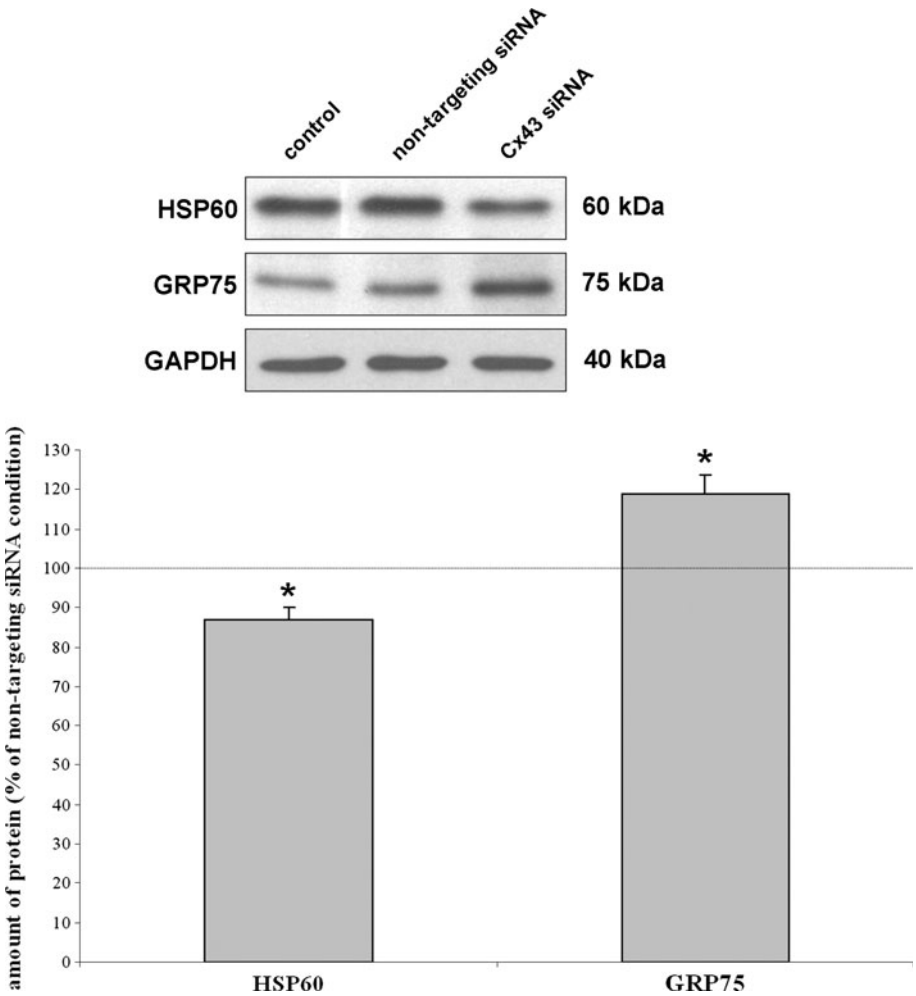

Fig. 4 Validation of the proteomics analysis. Freshly isolated rat hepatocytes were cultivated in a monolayer configuration. Twenty hours after plating, cell cultures were transfected with 100 nM non-targeting siRNA or Cx43 siRNA for 5 h as described in the "Materials and methods" section. Samples were taken 72 h post-transfection and subjected to immunoblot analysis as described in the "Materials and methods" section, using primary antibodies directed against HSP60 or GRP75 (Table 1). For semi-quantification of the results, marker signals in the Cx43 siRNA condition were normalized to the corresponding GAPDH signals and were expressed as percentage of the normalized marker signals in the non-targeting siRNA condition (indicated with a *dashed line*). Data were expressed as mean ± SD of seven independent experiments. Results were evaluated by one-way analysis of variance followed by post hoc Bonferroni tests. *Asterisks* indicate significant differences compared with the non-targeting siRNA condition ($*p < 0.05$)

positive targets of Cx43 siRNA, respectively, these proteins were selected for further scrutiny. Whole cell lysates were prepared of primary hepatocytes exposed to Cx43 siRNA and non-targeting siRNA and subjected to SDS–PAGE analysis. Following immunoblotting, membranes were incubated with primary antibodies directed against HSP60 and GRP75, and signals were semi-quantified through densitometry and by normalization against GAPDH. By doing so, treatment with Cx43 siRNA was found to significantly ($p < 0.05$) downregulate hepatocellular HSP60 protein levels to 87.3 ± 3.2 % of the non-targeting siRNA condition, while GRP75 quantities were raised to 119.0 ± 4.8 % (Fig. 4).

Effect of Cx43 suppression on the metabolome in primary hepatocyte cultures

Quenching of the Cx43 production caused no gross changes in the endogenous metabolism of the primary rat hepatocytes at the intracellular or the extracellular level as assessed by NMR-based metabolic profiling. Indeed, the cells appeared to exhibit normal metabolic behaviour in the Cx43 siRNA condition when compared to the control samples, as determined by measuring certain metabolites that represent important metabolic pathways or that are related to changes observed in the proteomics analysis (Table 2). Glucose (i.e., extracellular) and lactate and

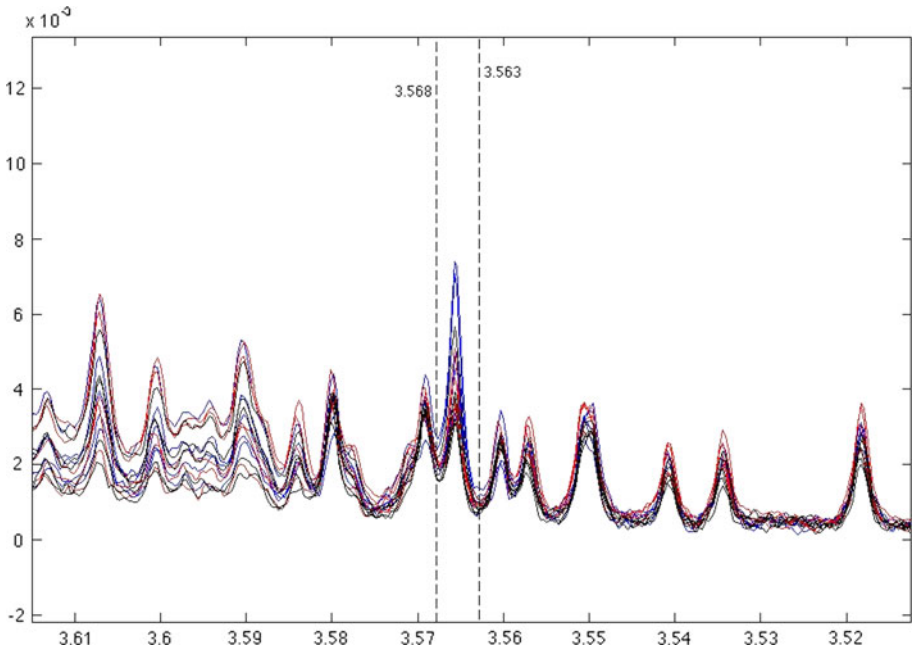

Fig. 5 Effect of Cx43 suppression on the metabolome in primary hepatocyte cultures. Freshly isolated rat hepatocytes were cultivated in a monolayer configuration. Twenty hours after plating, cell cultures were transfected with 100 nM non-targeting siRNA or Cx43 siRNA for 5 h as described in the "Materials and methods" section. Samples were taken 72 h post-transfection and subjected to metabolomics analysis as described in the "Materials and methods" section. Spectra of the intracellular fraction (i.e., aqueous soluble metabolites) are shown. The Cx43 siRNA condition, the non-targeting siRNA condition and untreated control conditions are presented in *blue*, *red* and *black*, respectively. The *dotted lines* show the area integrated to estimate the levels of glycine (3.563–3.568 ppm) (colour figure online)

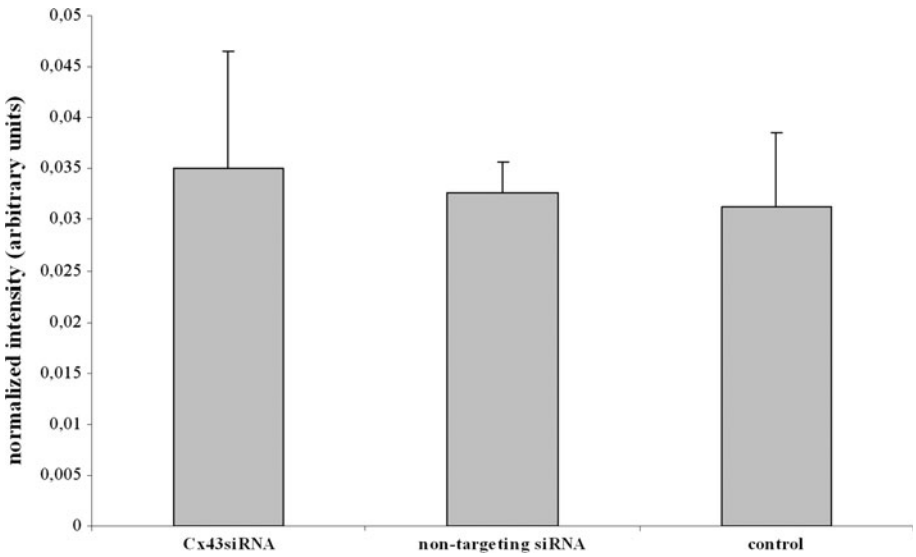

Fig. 6 Effect of Cx43 suppression on glycine levels in primary hepatocyte cultures. Freshly isolated rat hepatocytes were cultivated in a monolayer configuration. Twenty hours after plating, cell cultures were transfected with 100 nM non-targeting siRNA or Cx43 siRNA for 5 h as described in the "Materials and methods" section. Samples were taken 72 h post-transfection and subjected to metabolomics analysis as described in the "Materials and methods" section. Data shown have been normalized using the median fold change method of normalization and represent means per treatment group ± SD of three independent experiments. Results were evaluated by a means of a homoscedastic t-test

pyruvate (i.e., extracellular and intracellular) were monitored to investigate possible glycolytic modifications in the Cx43 siRNA condition. No significant changes associated with treatment were observed. Proteomics analysis showed a decrease in glutathione S-transferase levels by Cx43 siRNA (Table 2), but there was no concomitant alteration in the oxidized and reduced glutathione pool. Visual inspection of the normalized median spectra suggested a

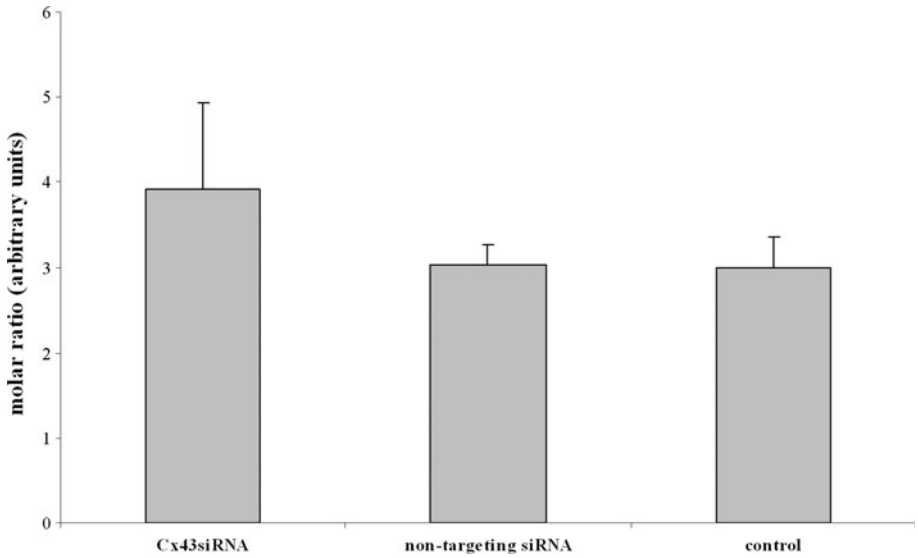

Fig. 7 Effect of Cx43 suppression on the glycine/pyruvate ratio in primary hepatocyte cultures. Freshly isolated rat hepatocytes were cultivated in a monolayer configuration. Twenty hours after plating, cell cultures were transfected with 100 nM non-targeting siRNA or Cx43 siRNA for 5 h as described in the "Materials and methods" section. Samples were taken 72 h post-transfection and subjected to metabolomics analysis as described in the "Materials and methods" section. Data shown represent mean glycine/pyruvate ratios per treatment group of three independent experiments. *Error bars* show 1 standard deviation, and results were evaluated by a means of a homoscedastic t-test

subtle increase in glycine levels associated with Cx43 silencing (Fig. 5). Direct integration of the glycine peak and statistical comparison of the Cx43 siRNA condition with the non-targeting siRNA and control conditions yielded p values approaching, but not reaching significance (i.e., 0.067 and 0.066, respectively) (Fig. 6). No significant differences were observed in the ratio between glycine and metabolically linked pyruvate, a strategy that does not rely on normalization (Fig. 7).

Discussion

Connexins have emerged as multifaceted signalling entities in the last decade, which basically relates to their assembly status and subcellular location. Connexins indeed reside not only at the cell plasma membrane surface, but also at other locations, such as in the nucleus and in mitochondria (Decrock et al. 2009; Vinken et al. 2006a, 2011a). The latter could be supported by the results of the current proteomics study, since a number of mitochondrial proteins were found to be affected by Cx43 silencing in primary hepatocyte cultures, including HSP60, GRP75, thiosulphate sulphurtransferase and ATP synthase. Yet, the functional relevance of mitochondrial Cx43 is a matter of debate. It has been postulated that Cx43 is part of a multiprotein complex that somehow controls mitochondrial homeostasis (Goubaeva et al. 2007). In this regard, mitochondrial Cx43 was recently shown to be required for respiratory complex I activity and hence for ATP production in cardiomyocytes (Boengler et al. 2012). Mitochondrial Cx43 could also form hemichannels and may affect potassium ion fluxes, a scenario that equally takes place in cardiomyocytes (Miro-Casas et al. 2009; Rottlaender et al. 2012). This function is reminiscent of apoptosis-regulating Bcl-2 family proteins. In fact, Cx43 was found to be an apoptosis regulator in a variety of cell types (Decrock et al. 2009; Goubaeva et al. 2007; Klee et al. 2011). Our group recently found that Cx43 signalling contributes to the occurrence of spontaneous apoptosis in freshly established cultures of primary hepatocytes, which partially depends on connexin hemichannel functionality (Vinken et al. 2012b). Although the results should be considered with caution and may be speculative, the outcome of the present global protein profiling study could further substantiate this observation. This may equally hold true for the metabolomic screening effort, which indicated a tendency for increased glycine levels upon knock-down of hepatocellular Cx43. Glycine is known to protect against ischaemia/reperfusion injury (Luntz et al. 2005), an event that is triggered during the isolation of primary hepatocytes from the liver and that negatively affects cell survival (Elaut et al. 2006). The mechanistic basis of the cytoprotective activity of glycine is unknown, but could involve stabilization of the plasma membrane preventing its failure and the onset of necrotic cell death (Kon et al. 2004) or inhibition of calcium signalling (Menger and Vollmar 2007), a cellular process in which connexin-related communication plays a prominent role (Decrock et al. 2011). In the same context, Cx43 suppression influenced the protein

levels of two biotransformation enzymes, namely glutathione S-transferase μ 2 and cytochrome P450 2C70. The former is an acknowledged detoxifier of electrophilic compounds, including those generated during oxidative stress (Raza 2011), which occurs in primary hepatocyte cultures as a result of ischaemia/reperfusion insults underwent during the two-step collagenase perfusion isolation procedure (Elaut et al. 2006). The biological meaning of the other protein changes induced by Cx43 silencing in primary hepatocyte cultures is less clear. In a similar proteomics study, using differential gel electrophoresis, it was found that Cx43 suppression mainly affects cytoskeletal proteins in primary mouse astrocyte cultures (Olk et al. 2010). Our results are in line with these findings, as both hepatocellular actin and keratin were downregulated by Cx43 siRNA treatment.

In conclusion, the present combined proteomics/metabolomics study revealed a number of cellular targets for Cx43 knock-down in primary hepatocyte cultures. At the same time, these data could be reconciled with the anticipated role of Cx43 signalling in cell death, in casu spontaneously occurring in primary hepatocyte cultures.

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